

Molecular Basis of the Inhibition of Human Aromatase (Estrogen Synthetase) by Flavone and Isoflavone Phytoestrogens: A Site-directed Mutagenesis Study

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Flavone and isoflavone phytoestrogens are plant chemicals and are known to be competitive inhibitors of cytochrome P450 aromatase with respect to the androgen substrate. Aromatase is the enzyme that converts androgen to estrogen; therefore, these plant chemicals are thought to be capable of modifying the estrogen level in women. In this study, the inhibition profiles of four flavones [chrysin (5,7-dihydroxyflavone), 7,8-dihydroxyflavone, baicalein (5,6,7-trihydroxyflavone), and galangin (3,5,7-trihydroxyflavone)], two isoflavones [genistein (4',5,7-trihydroxyisoflavone) and biochanin A (5,7-dihydroxy-4'-methoxyisoflavone)], one flavanone [naringenin (4',5,7-trihydroxyflavanone)], and one naphthoflavone (α -naphthoflavone) on the wild-type and six human aromatase mutants (I133Y, P308F, D309A, T310S, I395F, and I474Y) were determined. In combination with computer modeling, the binding characteristics and the structure requirement for flavone and isoflavone phytoestrogens to inhibit human aromatase were obtained. These compounds were found to bind to the active site of aromatase in an orientation in which rings A and C mimic rings D and C of the androgen substrate, respectively. This study also provides a molecular basis as to why isoflavones are significantly poorer inhibitors of aromatase than flavones. **Key words:** aromatase, aromatase inhibitors, computer modeling, cytochrome P450, estrogen synthesis, flavone, isoflavone, phytoestrogens, site-directed mutagenesis. *Environ Health Perspect* 106:85–92 (1998). [Online 21 January 1998] <http://ehpnet1.niehs.nih.gov/docs/1998/106p85-92kao/abstract.html>

As pointed out by Henderson (1) in 1993,

one of the most dramatic features of breast cancer is the disparity in incidence rates between highly westernized and nonwesternized countries. Women born and raised in the United States are at least five times as likely to get breast cancer as women born and raised in Japan. But Japanese women increase their risk if they live in rapidly westernizing Japanese cities or emigrate and live in the United States. As is true with coronary heart disease, differences in diet are thought to be a major underlying factor in the different incidence rates of breast cancer, particularly among postmenopausal women.

The hypothesis that differences in diet can change breast cancer and heart disease incidence in postmenopausal women is at least, in part, proposed based on results generated from studies on phytoestrogens. Phytoestrogens are plant chemicals that bind to the estrogen receptor and induce many components of estrogen action (2–10). The best known phytoestrogens are diphenolic chemicals that belong to the classes of flavonoids, isoflavonoids, and lignans (2–10). These compounds are thought to play a beneficial role in preventing breast cancer. They may function as antiestrogens or weak estrogens by competing with estrogens for binding to the estrogen receptor (ER). However, we feel that it is also possible that some of these compounds may act in an indirect fashion by inhibiting aromatase activity, resulting in a decrease in

the level of estrogen in women. This aspect may have been overlooked.

In estrogen-dependent breast tumors, estrogens induce the expression of peptide growth factors that are responsible for the proliferative responses of cancer cells (11,12). Aromatase, a cytochrome P450, is the enzyme synthesizing estrogens by catalyzing three consecutive hydroxylation reactions converting C19 androgens to aromatic C18 estrogenic steroids (Fig. 1). Because aromatase is the enzyme responsible for the synthesis of estrogens and because estrogens have a major effect on the development of breast cancer, an abnormal expression of aromatase in breast cancer cells or surrounding adipose stromal cells may have a significant influence in breast tumor maintenance and growth in breast cancer patients. Cell culture and nude mouse studies using aromatase-transfected MCF-7 and T-47D cells (13–15) have demonstrated that tumor aromatase can play a role in breast cancer maintenance and progression. It has also been suggested that *in situ* produced estrogen plays a more important role than circulating estradiol in breast tumor promotion (16,17). Estrogen synthesized by the tumor aromatase was shown to stimulate breast tumor growth in both an autocrine and a paracrine manner (15). Aromatase-inhibitor therapy is a second-line treatment for those who fail anti estrogen therapy. Twenty to 30% of the

patients who fail antiestrogen treatment respond to aromatase-inhibitor treatment. Phytoestrogens have been reported to be inhibitors of aromatase (18–22); therefore, these chemicals may function as chemopreventive agents by inhibiting aromatase/estrogen biosynthesis in postmenopausal women. In this study, we have examined the interaction of eight flavone and isoflavone phytoestrogens with human aromatase and its mutants by inhibition kinetic analysis and computer modeling. Some of these compounds such as chrysin (5,7-dihydroxyflavone) inhibit aromatase with K_i values similar to that of aminoglutethimide, an aromatase inhibitor used clinically to treat breast cancer. While phytoestrogens have been reported to be inhibitors of aromatase, the molecular basis of their inhibition of aromatase is not known.

During the past 5 years, using computer modeling and site-directed mutagenesis, we have evaluated a number of amino acid residues that are thought to be present in the active site and play a critical role for the binding of the androgen substrate (23–30). These aromatase mutants are very valuable in determining the binding characteristics of different aromatase inhibitors to the enzyme by examining how mutations in the active site of aromatase affect the binding of the inhibitors, as indicated by changes in their inhibition profiles (28–30). Recently, the binding characteristics of seven aromatase inhibitors were determined using the above

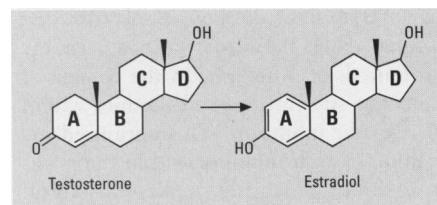


Figure 1. Aromatase converts androgens to estrogens.

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approaches (30). In this paper, we report the inhibition profiles of eight phytoestrogens on the wild-type aromatase and six mutants. By analyzing these results together with results generated previously with well-characterized aromatase inhibitors (28–30), information regarding the molecular basis of the inhibition of aromatase by phytoestrogens was obtained. This information is very important to further develop a strategy for prevention and treatment of breast cancer with plant chemicals such as phytoestrogens.

Materials and Methods

Materials. Unless otherwise indicated, the phytoestrogens tested in this study were purchased from Aldrich (Milwaukee, WI) or Calbiochem (La Jolla, CA). Dimethylsulfoxide was obtained from Mallinckrodt (Paris, Kentucky). [1β - ^3H (N)]-Androst-4-ene-3,17-dione was purchased from New England Nuclear (Boston, MA). Progesterone and NADPH (tetrasodium salt) were from Sigma Chemical Co. (St. Louis, MO).

Molecular modeling. The generation of a three-dimensional model of human aromatase has been described previously (31). The modeling of the binding of phytoestrogens in the active site was performed using the program BIOGRAF, a molecular modeling program marketed by Molecular Simulations, Inc. (Waltham, MA). The phytoestrogens were built using fragment libraries supplied with the program. Partial charges were assigned using the Q-Equilibrate algorithm. The initial structures, most of which were semirigid, were first energy minimized to a root-mean-square (RMS) force of less than 0.001. The resulting structures were subjected to 1 psec of molecular dynamics at 300 K, followed by conjugate gradient minimization to an RMS force of less than 0.001.

The minimized phytoestrogen molecules were then individually placed within the active site of the aromatase model such that overlap with the bound substrate was optimal. Each inhibitor was again energy minimized, but this time in the context of the active site (includes all residues within 10 Å of the inhibitor). This optimized the position of each inhibitor within the pocket, assuming that the pocket was rigid. Finally, we repeated the minimizations, assuming that residues within the active site can move to better accommodate the inhibitors (induced fit theory of molecular interaction) to see if the results better explain the inhibition binding data generated in our laboratory.

Stable expression and site-directed mutagenesis experiments. Aromatase mutant cDNAs were generated using a PCR-based mutagenesis method, which was

described in a previous publication (30). The mutant constructs were religated into the mammalian expression vector, pH β -Apr-1-Neo (32), through the engineered Sal I and Hind III restriction sites. The transfection procedure was the same as described previously (33) except that the selection process continued for 4 weeks with stepwise increases of G418 (Promega, Madison, WI) up to a final dosage at 680 $\mu\text{g}/\text{ml}$. Six mutants, I133Y, I395F, I474Y, P308F, D309A, and T310S, were generated by this method (23,24,26,30) and used in this study. The study was carried out with the wild type- and mutant-expressing Chinese hamster ovary (CHO) cells. The expression of these enzyme mutants in transfected CHO cells was confirmed by enzyme activity measurement and western blot analysis (or immunoprecipitation analysis) (23,24,26,30). A detailed discussion of the catalytic properties of these six mutants has been published (30).

Other experiments. The aromatase activity was determined by a $^3\text{H}_2\text{O}$ release assay, as described by Zhou et al. (33). This $^3\text{H}_2\text{O}$ release assay for human aromatase expressed in CHO cells was previously validated by the product isolation assay (33). For the aromatase inhibition study, phytoestrogens were dissolved in dimethylsulfoxide and added during the assays with 100 nM [1β - ^3H] androstenedione. In addition, the inhibition profile studies were performed using an in-cell method; the results produced by the in-cell method have been shown to be comparable to those determined using microsomal preparations of aromatase and its mutants (30). The K_i values for phytoestrogens were derived from Dixon plots ($1/v$ vs. $[I]$). These compounds were shown to be competitive inhibitors of aromatase with respect to the androgen substrate. The K_m values of the substrate for the wild type and mutant aromatase were needed for the calculation of K_i , and those can be found in Kao et al. (30).

Results and Discussion

Active site model of aromatase. Phytoestrogens such as flavones have been found to be competitive inhibitors of aromatase with respect to the substrate (18–22), suggesting that these compounds probably bind to the active site of the enzyme. Stronger evidence for the binding of these compounds to the active site of aromatase was obtained from difference absorption spectral studies. Kellis and Vickery (18) reported that α -naphthoflavone could displace androstenedione bound to human placental aromatase and induced a difference spectrum characteristic of the conversion of the heme-iron from a high-spin to a low-spin state. These authors

further suggested that the low-spin nature of α -naphthoflavone-enzyme complex indicates the presence of an additional axial ligand to the heme-iron, possibly the C4-keto group.

Both we (30) and Graham-Lorence et al. (34) have generated models of aromatase through an alignment of the amino acid sequence of the human aromatase with those of cytochromes P450cam, P450bm3, and P450terp [whose X-ray structures have been determined (35–37)] and homology modeling by computer. The two models from two laboratories are in a good agreement except in the region between the C and F helices. By examining the structures of cytochromes P450cam, P450bm3, and P450terp, most residues participating in the construction of the active site fall in three regions, including the sequence segment between the B' and C helices, the I helix, and the β 4-2 strand close to the carboxy terminus. Among the amino acid residues mutated in our laboratory, Ile-133 in the human aromatase is thought to be located in the region between the B' and C helices and predicted to form van der Waals interactions with the β face of the C and D rings of the steroid substrate (30).

In the I helix, there is a conserved threonine (Thr) residue in all the known cytochrome P450 structures except P450eryF (38), and Thr-310 in aromatase is the conserved residue. Thr-310 appears to be involved in stabilizing the distortion in the I helix by providing a hydrogen bond to the carbonyl group of alanine (Ala)-306; in addition, it may act as a proton donor during the redox reactions (30). Furthermore, the increased sensitivity of the mutant T310S to several nonsteroidal aromatase inhibitors suggests that, in the normal enzyme, the methyl group of Thr-310 may be involved in steric clashes with such inhibitors, somewhat reducing their binding affinity (30). The residue adjacent to the conserved threonine residue is an aspartic acid which is also highly conserved in cytochromes P450. Studies have suggested that aspartic acid (Asp)-309 is an important residue in the active site of aromatase (24,34). An Asp-309 to Ala mutation could cause a partial collapse in the active site cavity that reduces the ability of all the studied inhibitors, except aminogluthethimide (AG), to bind to aromatase (30). AG is smaller than the androgen substrate, and the enhanced inhibition of D309A may result from a tighter binding of AG in an active site cavity of reduced volume. In addition, a characteristic of cytochrome P450 structures is the distortion in the central region of the I helix with the formation of a cleft in the region of the heme iron. In the cytochrome P450cam-based model of aromatase, proline (Pro)-308 forms part of this cleft. Interestingly, the aromatase mutant

P308F was found to be more sensitive to treatment by two steroidal inhibitors [4-hydroxyandrostenedione (4-OHA) and 7α -(4'-amino)phenylthio-1,4-androstadiene-3,17-dione (7α APTADD)] than the wild-type enzyme (30).

Isoleucine (Ile)-474 in the carboxy terminus of aromatase corresponds to Ile-395, an active site residue in cytochrome P450cam, and is thought to be situated around the lower edge of the B ring of the androgen substrate in aromatase (30). Ile-395 is in a region (residues 380–402) conserved among aromatase of all species analyzed so far. The K_m value of androstenedione for I395F was found to be significantly larger than that of the wild-type aromatase (30), suggesting that the mutation reduces the binding affinity of the substrate.

To study how flavone phytoestrogens interact with aromatase, we investigated the interaction of eight phytoestrogens, including both flavones and isoflavones, with the wild-type aromatase and six well-characterized mutants, i.e., I133Y, P308F, D309A, T310S,

I395F, and I474Y. Previous studies from our laboratory have found that these mutations significantly affect the binding of the substrates and inhibitors of the enzyme (23,24,26,30).

Inhibition of aromatase by phytoestrogens. Figure 2 shows a computer-generated model of the active site region of aromatase along with the substrate androstenedione. The positions of Ile-133, Pro-308, Asp-309, Thr-310, Ile-395, and Ile-474 are highlighted.

The inhibition study involved four flavones [chrysin (5,7-dihydroxyflavone), 7,8-dihydroxyflavone, baicalein (5,6,7-trihydroxyflavone), and galangin (3,5,7-trihydroxyflavone; also classified as a flavanol)], two isoflavones [genistein (4',5,7-trihydroxyisoflavone) and biochanin A (5,7-dihydroxy-4'-methoxyisoflavone)], one flavanone [naringenin (4',5,7-trihydroxyflavanone)], and one naphthoflavone (α -naphthoflavone). The structures of eight phytoestrogens are shown in Figure 3. The potency of these compounds as aromatase inhibitors

has been determined according to their K_i values: α -naphthoflavone ($2.2 \pm 0.1 \mu\text{M}$) > chrysin ($2.6 \pm 0.1 \mu\text{M}$) > naringenin ($5.1 \pm 0.2 \mu\text{M}$) > 7,8-dihydroxyflavone ($10 \pm 1 \mu\text{M}$) > biochanin A ($12 \pm 5 \mu\text{M}$) > galangin ($95 \pm 28 \mu\text{M}$) > genistein ($123 \pm 8 \mu\text{M}$). No inhibition by baicalein was observed with concentrations up to $50 \mu\text{M}$. α -Naphthoflavone is the best aromatase inhibitor among all the phytoestrogens studied [agreeing with findings from other laboratories (17,20)], and its K_i values are not different from those of the aromatase inhibitor AG ($1.37 \mu\text{M}$). Isoflavones are less effective than flavones in inhibiting aromatase.

We evaluated the interaction of phytoestrogens and aromatase mutants by comparing their K_i values. The K_i values of seven phytoestrogens to six aromatase mutants are shown in Table 1. Among the four flavones tested, chrysin is the best inhibitor. We modeled the binding of chrysin to the active site of aromatase based on the model for an aromatase inhibitor, vorozole, because of the resemblance in inhibition profiles with the mutants (30). We (30) and Graham-Lorence et al. (34) predicted an orientation of vorozole in which the 1-methylbenzotriazole mimics the C and D rings of the substrate and the *p*-chlorophenyl substituent is directed toward Asp-309. Furthermore, on the basis of the mutagenesis data, we predict

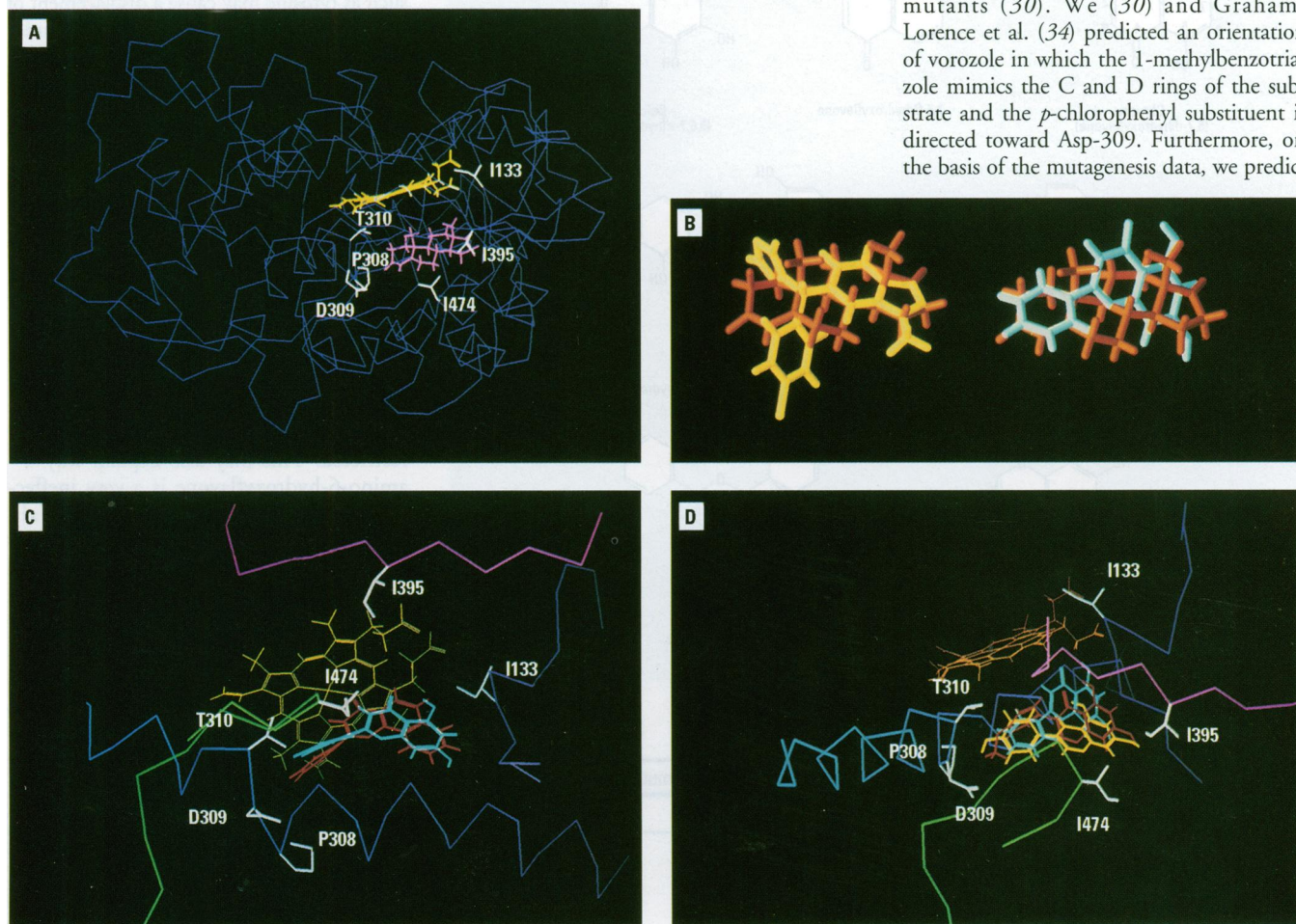


Figure 2. Models of the predicted orientations of phytoestrogens in the active site of aromatase. (A) A computer-generated model of the active site region of aromatase along with the substrate androstenedione. (B) The predicted orientation of chrysin (cyan), vorozole (yellow), and androstenedione (orange), in the active site of aromatase. (C) Predicted orientations of α -naphthoflavone (red) along with chrysin (cyan). (D) Predicted orientations of biochanin A (red) and genistein (yellow). Abbreviations: T310, threonine 310; I133, isoleucine 133; I395, isoleucine 395; I474, isoleucine 474; P308, proline 308; D309, aspartic acid 309.

an orientation in which the A and C rings of flavone mimic rings C and D of a steroid (see Fig. 2B). By doing so, the *p*-phenyl substituent (i.e., ring B) of chrysin is oriented in a similar position to that of the substrate's ring A and is directed towards Asp 309. The *p*-phenyl substituent tilts approximately 30° in reference to rings A and C of the flavone (see Fig. 2B). The C4-keto group of chrysin points towards the heme prosthetic group (Fig. 2C). Our computer modeling analysis places the C4-keto group of flavones in an area for the angular C19 of the androgen steroid. Ibrahim and Abul-Hajj (19) reported that a reduction of the C4-keto group to a hydroxyl group greatly decreased the inhibitory potency. As indicated above, the low-spin nature of α -naphthoflavone-enzyme complex led Kellis and Vickery (18) to suggest the presence of an additional axial ligand to the heme-iron, possibly the

C4-keto group of the flavone. Such a model also explains why polyphenols such as epicatechin and epigallocatechin, which are present in tea and do not have a keto group at the corresponding position, are not capable of inhibiting aromatase (Kao et al., unpublished observations; these compounds were provided by C. S. Yang from the College of Pharmacy, Rutgers University).

It has been reported that 5-hydroxyflavone is a less potent inhibitor of aromatase than flavone (19). The 5-hydroxy group probably forms an intramolecular hydrogen bonding with the 4-keto group [indicated by nuclear magnetic resonance (NMR) analysis (39)], weakening the interaction of the 4-keto group with the heme. In a recently completed experiment, 7-hydroxyflavone was found to be four times more potent than chrysin (Kao et al., unpublished results). We feel that chrysin

resembles a seco-steroid that has an opened B-ring and a homo D-ring. As suggested previously (30), Pro-308 to Phe and Asp-309 to Ala mutations modify the size of the active site pocket, thus preventing the binding of chrysin as indicated by increases in the K_i values (Table 1). Furthermore, the increased sensitivity of T310S to chrysin suggests that in the normal enzyme this methyl group may be involved in steric clashes with the inhibitor, reducing its binding affinity somewhat. According to our model, Ile-474 appears to be near the beta-face and lower edge of chrysin's *p*-phenyl ring. The increased potency for I474Y might be due to favorable aromatic-aromatic interactions between the phenyl substituent and the mutated residue. Ile-395 is assigned to lie at the far end of the hydrophobic pocket, and an aromatic-aromatic interaction may also explain the increased potency by the mutation Ile-395 to Phe. Ile-133 has been proposed to be situated close to the heme in the current model. Replacement by a bulkier residue such as tyrosine may cause a displacement of the heme, probably exerting an indirect effect on the bindings of flavones. A conformational change in the active site by converting Ile-133 to Tyr increases the relative binding affinity of chrysin, as indicated by a decrease in the K_i value.

Baicalein, which differs from chrysin by having an additional hydroxyl group at the C-6 position, could not inhibit the wild-type aromatase with concentration up to 50 μ M. Ibrahim and Abul-Hajj (19) have reported that 6-hydroxyflavone is a significantly weaker inhibitor of aromatase than flavone. These findings indicate that the region around the C-6 position in the active site of aromatase must be very restricted. This may also explain why 4'-amino-6-hydroxyflavone is a very ineffective inhibitor of aromatase (Kao et al., unpublished results). Because baicalein was found to be a very poor inhibitor for the wild-type aromatase, we did not pursue the study with mutants.

While 7,8-dihydroxyflavone was found to be a less potent inhibitor than chrysin, the inhibition profiles of this compound were very similar to those of chrysin except

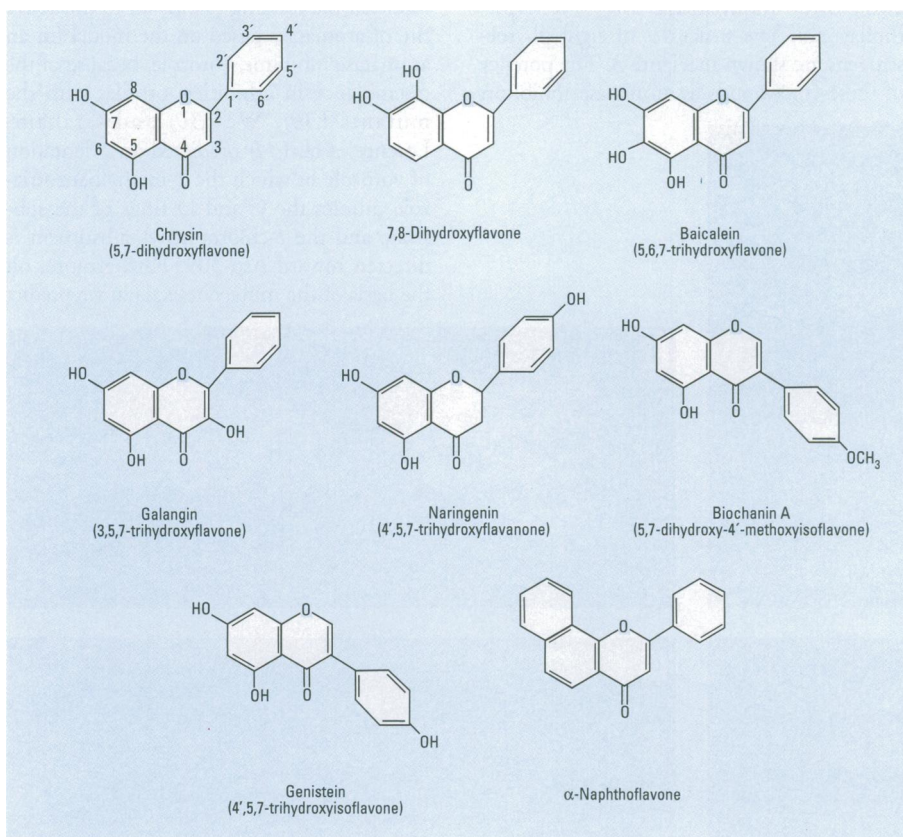


Figure 3. Structures of eight phytoestrogens.

Table 1. K_i values^a of seven phytoestrogens to the wild-type aromatase and six mutants

Enzyme form	Chrysin	7,8-Dihydroxyflavone	Galangin	α -Naphthoflavone	Naringenin	Genistein	Biochanin A
Wild type	2.6 \pm 0.1	10 \pm 1	95 \pm 28	2.2 \pm 0.1	5.1 \pm 0.2	123 \pm 8	12 \pm 5
I133Y	0.3 \pm 0.1	86 \pm 25	3.1 \pm 1.3	0.8 \pm 0.1	4.2 \pm 1.3	16 \pm 2	5.9 \pm 1.1
I395F	0.6 \pm 0.1	1.3 \pm 0.2	4.7 \pm 1.9	0.5 \pm 0.1	1.0 \pm 0.2	30 \pm 7	7.9 \pm 2.7
I474Y	0.06 \pm 0.02	0.3 \pm 0.1	5.9 \pm 1.1	1.2 \pm 0.1	0.5 \pm 0.1	24 \pm 2	1.0 \pm 0.1
P308F	3.2 \pm 0.3	13 \pm 2	52 \pm 12	0.6 \pm 0.1	7.5 \pm 0.3	42 \pm 19	24 \pm 4
D309A	13 \pm 3	40 \pm 21	22 \pm 4	2.8 \pm 0.5	40 \pm 7	92 \pm 11	161 \pm 24
T310S	1.4 \pm 0.2	1.7 \pm 0.5	33 \pm 2	2.7 \pm 0.8	1.7 \pm 0.3	90 \pm 18	31 \pm 9

^aShown in μ M.

with the mutant I133Y (Table 1). The results indicate that the binding affinity decreases by having a hydroxyl group at the C-8 instead of the C-5 position. Furthermore, it is proposed that the presence of a hydroxyl group at the C-8 position may push the A ring of the flavone closer to heme or Ile-133, resulting in a great increase of the resistance of I133Y to this flavone. As indicated in Figure 4, computer modeling suggests another possible orientation for the binding of flavone to the active site of aromatase, an orientation in which rings A and C of flavone mimics rings A and B of a steroid and the *p*-phenyl substituent is directed towards Ile-133. However, this binding orientation is thought to be unfavorable because the mutant I133Y has been found to be more resistant to 7,8-dihydroxyflavone than the wild-type aromatase, whereas the same mutant is more sensitive to chrysin than the wild-type aromatase. Furthermore, in the latter orientation, the C4-keto group would be away from the heme, making it difficult to explain the results associated with this substituent (18,19). Because 7,8-dihydroxyflavone has the same *p*-phenyl substituent as chrysin, the relative responses to P308F, D309A, T310S, and I474Y were found to be similar to chrysin.

The results for galangin indicate that the presence of a hydroxyl group at position 3 of the flavone significantly reduces its ability to bind to aromatase. Computer modeling has revealed that the 3-hydroxyl group is pointing to the I-helix and affects the orientation of the phenyl substituent at the C-2 position and may also interfere with the interaction of the 4-keto group with the heme. Two types of inhibition profiles were observed for this compound; three mutants with changes located on the distal helix (P308F, D309A, and T310S) exhibited regular dose-dependent profiles, while the other three mutants as well as the wild-type enzymes exhibited unusual biphasic profiles (Fig. 5). The 3-hydroxyl group of galangin and Thr-310 of the enzyme are thought to be on the same side of the beta-face of the flavone's ring-C. If a

larger accessible volume of the binding cavity is obtained by removing the methyl group of threonine at position 310, galangin could bind better in the resulting active site. Our inhibition studies have shown that T310S is more sensitive to galangin than the wild-type aromatase at concentrations less than 150 μ M (Fig. 5). Contrary to chrysin and other flavones, galangin binds relatively better to the mutant D309A than to the wild-type enzyme, suggesting that galangin fits better in the active site with a Asp-309 to Ala mutation. The nature of the biphasic inhibition profiles is not known. There is a possibility that galangin interacts with other proteins in CHO cells. However, an interaction with other proteins cannot explain why biphasic inhibition profiles were found for some but not all mutant forms of the enzyme. At the present time, we cannot explain these results adequately. As predicted from the computer model, the increased potency of galangin for I474Y may be due to a favorable aromatic-aromatic interaction between the *p*-phenyl substituent and the mutated residue. Since both chrysin and galangin bear the same A ring, the relative potency of I133Y to wild-type aromatase exerted by these compounds has been found to be comparable in the two compounds.

α -Naphthoflavone, but not β -naphthoflavone, is a good inhibitor of aromatase. α -Naphthoflavone has an additional benzoring at the C-7 and C-8 positions of ring-A of flavone. When superimposed over the structure of chrysin, the C4-keto group of α -naphthoflavone points closer to the heme, implying a better fit in the active site of aromatase (see Fig. 2C). A smaller K_i value of α -naphthoflavone for P308F than the wild-type aromatase suggests further that the binding orientation of α -naphthoflavone is slightly different from that of chrysin. As discussed above, Kellis and Vickery (18) observed that removal of the C4-keto group of 7,8-benzoflavone's pyrone C-ring to a furan ring totally diminished the inhibition ability, again indicating that the 4-keto group is essential for the

inhibition of aromatase. β -Naphthoflavone has a structure very different from α -naphthoflavone and does not fit in the active site of the enzyme; this agrees with our inhibition experiment, which indicates that β -naphthoflavone is a very poor inhibitor (results not shown).

Naringenin is slightly less potent than chrysin, but the inhibition profiles of naringenin were found to be similar to those of chrysin. These results indicate that a conversion of C2, C3 double bond to a single bond has only a small effect on its binding when compared to chrysin. In fact, the C2 atom in naringenin is a chiral center. Our computer modeling has revealed that only the *S*-configuration isomer can bind to the active site. Therefore, the observed K_i values might originate from a racemic mixture and the actual K_i values for the *S*-configuration isomer may be lower than those shown in Table 1. However, the presence of a single bond between the C-2 and C-3 positions does slightly modify the conformation of ring C (as shown by the computer modeling), explaining why there was an increase in the K_i value for I133Y by comparing it to that of chrysin. On the other hand, the presence of a 4'-hydroxyl group has only a small influence on the binding of the compound, as indicated by similar inhibitory profiles as

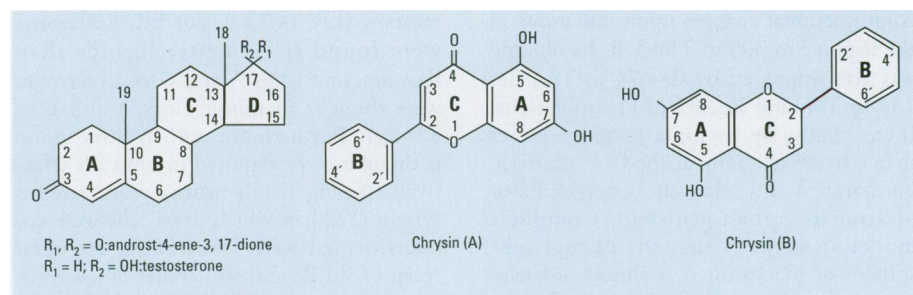


Figure 4. Two possible orientations of flavones that can fit into the proposed active site pocket of aromatase.

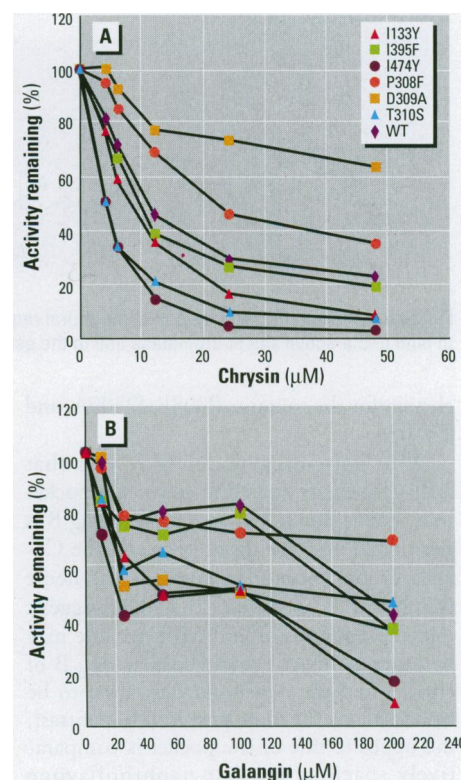


Figure 5. Inhibition profiles of wild-type (WT) aromatase and six mutants by chrysin (A) and galangin (B) as determined by the $^3\text{H}_2\text{O}$ release assay (see Materials and Methods).

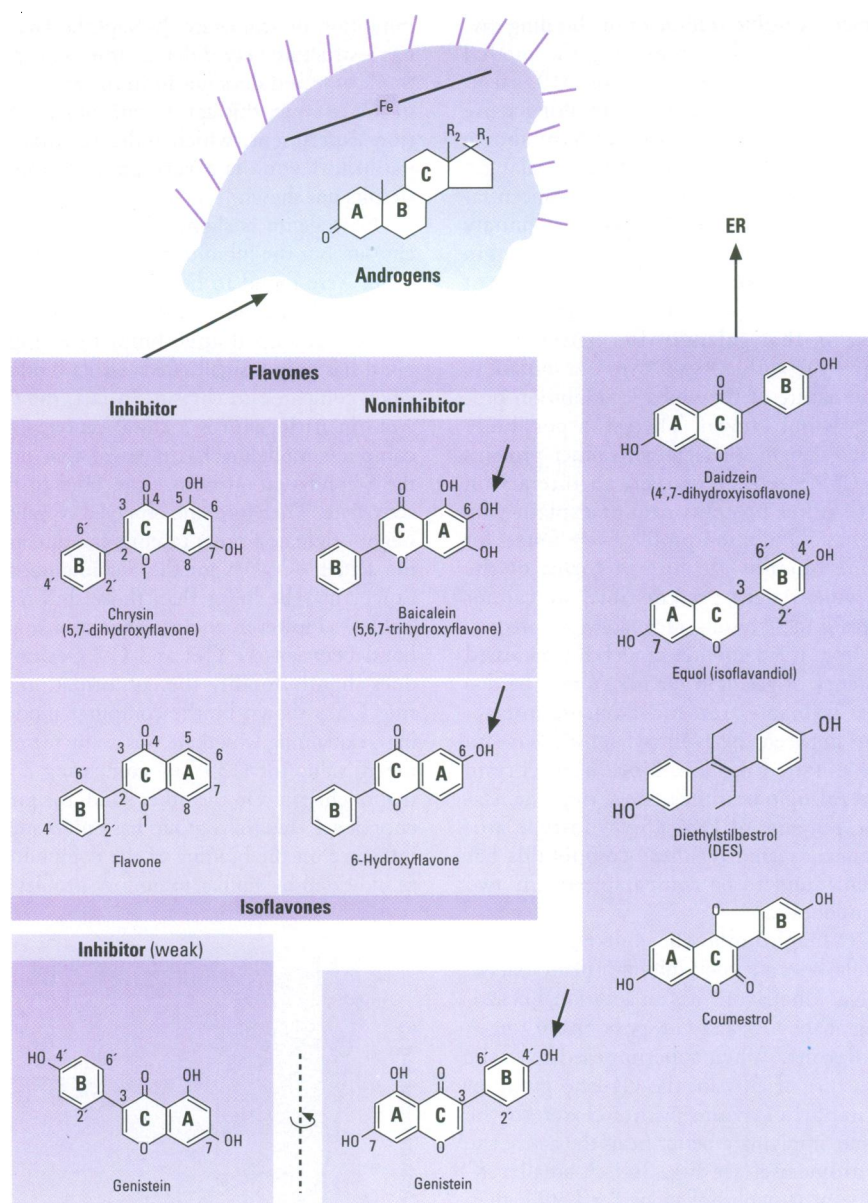


Figure 6. A schematic presentation of structural requirements for flavone and isoflavone phytoestrogens to bind to the active site of aromatase and to the estrogen receptor (ER).

chrysin for the mutants P308F, D309A, and T310S.

Our computer modeling reveals that isoflavones may enter the active site pocket in a similar way as flavones in that ring B is positioned near the distal helix and the C4-keto group is pointing towards the heme (see Fig. 2D). Computer modeling suggests that the pocket on the distal helix side may be more compact or rigid so that ring B of chrysin and the two isoflavones have to be held at about the same position. In contrast, the opposite side of the pocket is comparatively spacious (e.g., α -naphthoflavone binds stronger in the active site of aromatase) and therefore allows the benzopyrone rings of these compounds relatively free movement. Among these compounds,

the C4-keto group of genistein is the farthest from the heme as seen in the model (Fig. 2D), in agreement with its poor inhibitory potency. The results obtained from inhibition profile studies indicate that the binding of genistein is improved by conformational changes upon mutations in the region Pro-308 to Thr-310. Its binding was also improved by Ile-474 to Tyr, Ile-133 to Tyr, and Ile-395 to Phe mutations. Interestingly, by having a methoxy rather than a hydroxyl group at the C-4' position, biochanin A is a relatively better inhibitor of aromatase than genistein. Computer modeling suggests that the phenyl substituent of biochanin A is almost 90° relative to the plane of chrysin's ring B (Fig. 2D), explaining why the binding affinity of

biochanin A was found to decrease in mutants P308F, D309A, and T310S.

Flavone structure-activity studies from other laboratories (18–21) and our present study have revealed the structural features for flavone and isoflavone phytoestrogens to inhibit aromatase. By evaluating the interaction of eight phytoestrogens with the wild-type aromatase and six mutants by computer modeling and inhibition profile analysis, we have obtained further information regarding how phytoestrogens bind to the active site of aromatase. Figure 6 summarizes our current knowledge as to how flavones bind to the active site of aromatase. These compounds bind to the active site in an orientation such that their A and C rings mimic rings D and C of the steroid, respectively. The C4-keto group points to the heme prosthetic group. The presence of a 5-hydroxy group reduces the binding affinity, probably by forming a hydrogen bond with the 4-keto group. 7-Hydroxy- and 8-hydroxy-containing flavones are reasonable inhibitors of aromatase. The C-6 position of flavones is situated in a very restricted area; therefore, any flavones that have a C-6 substituent cannot bind to the enzyme. Furthermore, a 3-hydroxy group significantly changes the orientation of ring B, resulting in a large decrease in the ability to inhibit aromatase. Isoflavones such as genistein and daidzein have the 4'-hydroxyphenyl group at position C-3, greatly reducing the ability to bind and inhibit aromatase. These isoflavones cannot bind to aromatase in an orientation in which rings A and C mimic rings A and B of the steroid because the 4'-hydroxy group will be situated in the area equivalent to the C-6 position in flavones. While it is possible that isoflavones without the 4'-hydroxy group may inhibit aromatase, such compounds are not yet available for our investigation. Ongoing research in this laboratory will evaluate the effect of the hydroxy substituents of ring B on the ability to inhibit aromatase.

The binding orientation of flavones and isoflavones in relation to steroids in the active site of aromatase is different to that proposed for their binding to the estrogen receptor (ER) (40,41). For ER, isoflavones were found to be better ligands than flavones, and rings A and C of isoflavones were thought to mimic rings A and B of estrogen. Furthermore, a 4'-hydroxy group is thought to be required to bind ER effectively (see Fig. 6). By using a yeast estrogen system (YES) in which yeast cells were co-transformed with the human estrogen receptor (hER) and two copies of an estrogen response element (ERE) linked to the lacZ gene, Collins et al. (42) recently

examined the agonist/antagonist activity of various flavone and isoflavone phytoestrogens. The abilities of these compounds in competing with [^3H] 17 β -estradiol for binding to the recombinant hER were also measured. The IC_{50} values (i.e., the concentration of the ligand competitor at which the binding of radiolabeled 17 β -estradiol to the hER was reduced to 50%) for coumestrol, genistein, biochanin A, chrysin, and naringenin were determined as 0.01 μM , 2.0 μM , 6.0 μM , 33 μM , and 45 μM , respectively (42). The results indicate that isoflavones such as genistein and biochanin A bind to ER 5–10 times stronger than flavones such as chrysin and flavanones such as naringenin. Therefore, in the present study, we reveal the different structural features for phytoestrogens binding to aromatase and estrogen receptor.

We feel that this investigation has demonstrated the utility of a combined approach, which involves computer modeling, site-directed mutagenesis, and inhibitor profile analysis using structurally related inhibitors in a molecular characterization of an inhibitor binding site. Since we have found that flavones are known to be competitive inhibitors of NAD(P)H:quinone acceptor oxidoreductase with respect to NAD(P)H (43), it is possible that these compounds also inhibit NADPH-cytochrome P450 reductase. However, we feel that this possibility is unlikely, or at least the cytochrome P450 reductase inhibition does not play a major role here because the inhibition profiles do vary for different aromatase mutants.

Phytoestrogens such as flavones, isoflavones, and flavanones are widely present in the plant kingdom with the exception of algae, fungi, and hornworts (44). These compounds are found in all parts of the higher plants: roots, stems, leaves, flowers, pollen, fruit, seeds, wood, and bark. For example, chrysin is commonly found in flowers such as the passion flower (45); the genus *Citrus* is especially rich in flavanones such as naringenin (46); and genistein and biochanin A are known to be present in soybean extracts (47). Adlercreutz et al. (48) reported that in some vegetarian women plasma genistein concentrations could be as high as 0.1 $\mu\text{mol/l}$ as determined by reverse isotope dilution gas chromatography-mass spectrometry. Adlercreutz et al. (49) have also estimated that the plasma level of genistein in people on a high soy-containing diet is 1–4 $\mu\text{mol/l}$. Barnes (50) has made the following calculation: A person who consumes 35 g/day of soybeans (the average amount consumed by the Taiwanese) has an intake of ~50 μg (185 μmol) of genistein; if we assume that genistein equilibrates with total

body water (56 liters), the equilibrium plasma concentration would be 3.3 $\mu\text{mol/l}$. While there has not been an extensive examination of the concentrations of various phytoestrogens in our body fluids, it is estimated that the overall concentration of all flavones and isoflavones in circulation is in the micromolar range. In our study, some of the compounds are found to inhibit aromatase with K_i values in the micromolar range.

The present study is an *in vitro* study. The effects could be different *in vivo* due to metabolic conversion processes. Cell culture and animal studies including these chemicals have been initiated in our laboratory. While the *in vivo* studies are critical for a better understanding of the action of phytoestrogens on estrogen biosynthesis, the present study provides a molecular basis regarding the binding nature and structural requirements of phytochemicals to inhibit aromatase.

Since the Women's Health Initiative is carrying out a study to test the hypothesis that a diet high in fruits and vegetables will lower breast cancer incidence in postmenopausal women, we anticipate that our study of phytoestrogens will generate information regarding which phytoestrogens are able to control estrogen biosynthesis. This will help to determine which fruits and vegetables, those containing proper phytoestrogens, should be included in a diet for postmenopausal women.

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